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The impact of erythropoietin and female sex hormones in chronic hypoxic mice

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To Bela

To my family

To Brave-guy

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I. ABBREVIATIONS

AMS	Acute mountain sickness
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CB	Carotid body
CMS	Chronic mountain sickness
CO_2	Carbon dioxide
eNOS	Endothelial nitric oxide synthase
Epo	Erythropoietin
EpoR	Erythropoietin receptor
FIO_2	Inspired oxygen fraction
fR	Respiratory frequency
HDV	Hypoxic ventilatory depression
HIF	Hypoxia-inducible factor
HVR	Hypoxic ventilatory response
K^+	Potassium
NO	Nitric oxide
NTS	Nucleus tractus solitarious
O_2	Oxygen
PaO_2	Oxygen partial pressure
preBötC	Pre Bötzinger complex
rhEpo	Recombinant human erythropoietin
RIA	Radio immuno assay
Tg6	Transgenic mouse line 6, Epo overexpression in brain and lung
Tg21	Transgenic mouse line 21, Epo overexpression in brain only
TH	Thyrosine hydroxylase
VAH	Ventilatory acclimatization to hypoxia
$\dot{V}CO_2$	Carbon dioxide production
$\dot{V}O_2$	Oxygen consumption
VRG	Ventral respiratory group
VE	Ventilation
VT	Tidal volume
WT	Wildtype

1. SUMMARY

Erythropoietin (Epo) plays a critical role in the acclimatization process to hypoxia. This is due to renal Epo that activates erythropoiesis and in turn augments the oxygen carrying capacity of arterial blood. Additionally, cerebral and plasma Epo interact with the central and peripheral respiratory centers, respectively, thereby increasing minute ventilation. Knowing that women cope better than men with environmental hypoxia, we analyzed the ventilatory acclimatization to hypoxia (VAH) in transgenic female mice termed Tg6 that have elevated Epo levels in brain and plasma. Unexpectedly, we observed that after chronic hypoxia (3 days at 10% O_2) VAH was entirely abolished in these Epo-overexpressing Tg6 females but not in the WT controls. As this phenomenon occurred exclusively in female but not male mice, we suspected the involvement of sexual female steroids in hypoxic ventilation. Indeed, plasma measurements revealed that estradiol was 3-folds higher in transgenic mice than in corresponding WT female siblings while progesterone was not altered. Moreover, ventilatory measurements of ovarectomized Tg6 mice exposed to chronic hypoxia showed a recovery from the lost VAH. These results imply that under chronic hypoxia Epo interacts with sexual female hormones, and that this interaction blunts the VAH. To define whether this interaction occurs at central or peripheral respiratory centers, a bilateral transection of the carotid sinus nerves that uncouples the carotid bodies from the brainstem (chemodenervation) was performed. The observation that chemodenervated transgenic and WT females showed similar VAH implies that the interaction between Epo and sexual female steroids occurs at the level of the carotid body. This notion was confirmed by additional experiments in which measurements of the ventilatory response to chronic hypoxia and to hyperoxia (Dejours test) of untreated, ovarectomized and hormonally reconstituted ovarectomized Tg6 and WT female mice were performed. In summary, our results show that sexual female steroid interaction and Epo impair the VAH, and imply that this interaction occurs specifically in female carotid bodies exposed to chronic hypoxia. These findings are of central importance in the physiological response to hypoxia providing new insights into disease pathogenesis and to generate novel therapeutic approaches.

2. ZUSAMMENFASSUNG

Erythropoietin (Epo) spielt eine Schlüsselrolle im ventilatorischen Anpassungsprozess an Hypoxie. Einerseits aktiviert die renale Epo Freisetzung die Erythropoiese, was wiederum zu einer Erhöhung der Sauerstofftransportkapazität des Blutes führt, andererseits interagiert cerebrales und plasma Epo mit den zentralen und peripheren Atmungszentren, was zu einer erhöhten Ventilation führt. Da seit langem bekannt ist, dass Frauen sich besser an eine hypoxische Umgebung anpassen können als Männer, untersuchten wir die ventilatorische Anpassung an Hypoxie (VAH) weiblicher transgener Mäuse (Tg6), die eine konstitutiv erhöhte Epo Konzentrationen im Gehirn und im Blut aufweisen. Unerwarteterweise zeigten die Tg6 Weibchen nach dreitägiger Hypoxie (10%O_2) keinerlei VAH mehr. Da dieses Phänomen ausschliesslich in weiblichen und nicht in männlichen Mäusen beobachtet werden konnte, lag die Vermutung nahe, dass weibliche Sexualhormone an diesem Prozess beteiligt sind. Analysen der Plasmakonzentrationen der weiblichen Sexualhormone ergaben tatsächlich, dass Tg6 Weibchen dreimal höhere Estradiolplasmaspiegel aufwiesen als gleichaltrige WT Weibchen. Zusätzlich zeigten Atmungsmessungen ovarektomierter Tg6 Weibchen, dass diese nach der Entfernung der Gonaden plötzlich zur VAH fähig waren. Diese Daten deuten darauf hin, dass in chronischer Hypoxie Epo mit weiblichen Sexualhormonen interagiert und dass diese Interaktion zu einer Verhinderung der VAH führt. Um festzustellen ob diese Interaktion in zentralen oder peripheren Atmungszentren stattfindet, wurde eine bilaterale Durchtrennung der Sinusnerven der Karotis durchgeführt (Chemodenervation) um eine Signaltrennung der Karotiskörperchen vom Hirnstamm zu erreichen. Da nach der Chemodenervation keinerlei Unterschiede bezüglich VAH zwischen Tg6 und WT Weibchen mehr feststellbar waren, deutet dieses Resultat darauf hin, dass die Interaktion zwischen Epo und den weiblichen Sexualhormonen in den Karotiskörperchen stattfindet. Um diese Beobachtung zu bestätigen wurden zusätzliche Experimente durchgeführt, in denen die ventilatorische Antwort auf chronische Hypoxie und auf Hyperoxie gemessen wurde. Diese Experimente wurden mit unbehandelten, ovarektomierten und ovarektomierten Weibchen nach Hormonersatz durchgeführt. Zusammengefasst zeigen unsere Resultate, dass die Interaktion zwischen Epo und weiblichen Sexualhormonen die VAH verhindert und dass diese Interaktion in weiblichen

Karotiskörperchen in chronischer Hypoxie stattfindet. Diese Erkenntnisse sind von grosser Wichtigkeit für das Verständnis der physiologischen Antwort auf Hypoxie, geben neue Einblicke in Krankheitspathogenesen und ermöglichen neue therapeutische Ansätze.

INTRODUCTION

Several questions regarding ventilatory disorders related to hypoxic conditions remain to be elucidated. As such, it is still not known why some individuals react in a pathological way to acute exposure to hypoxia (acute mountain sickness, AMS) (Jacobson 1988; Bartsch, Bailey et al. 2004; Clarke 2006; Schoene 2008), or why some high altitude natives suddenly get desadapted to the hypoxic environment and develop chronic mountain sickness (CMS) (Monge and Whittembury 1976; Reeves and Leon-Velarde 2004; Wu 2005). To answer these questions it is important to understand the physiological processes that allow acclimatization to hypoxia (ventilatory acclimatization to hypoxia, VAH). Among other mechanisms these processes include increased ventilation, increased oxygen (O_2) carrying capacity of the blood via an increased erythropoietin production in the kidney (Fig. 1), higher tissue vascularisation and an augmentation of the number of mitochondria (Namiki, Brogi et al. 1995; Beall 2007; Paffett and Walker 2007).

The “classical” function of erythropoietin (Epo) is to increase the number of red blood cells. Recently however, it was found that brain and plasma Epo also control ventilation under hypoxic conditions (Fig.1) (Soliz, Joseph et al. 2005). Moreover it was observed that Epo influences hypoxic ventilation in a gender dependent manner. Female mice are more sensitive to Epo in acute hypoxia than males what results in a higher hypoxic ventilatory response (HVR) of female mice compared to males (Soliz, Thomsen et al. 2009). In contrast, when exposed to chronic hypoxia, Epo paradoxically impairs the VAH of female mice while in male mice Epo does not show any negative effect on the VAH. Accordingly, the aim of the present study was to characterize the acclimatization to hypoxia of female mice that constitutively overexpress Epo in brain and lung in an O_2-independent manner. We demonstrated that Epo has a deleterious interaction with female sex steroids upon exposure to chronic hypoxia and that this interaction occurs on the level of peripheral chemoreceptors, e.g. carotid bodies.

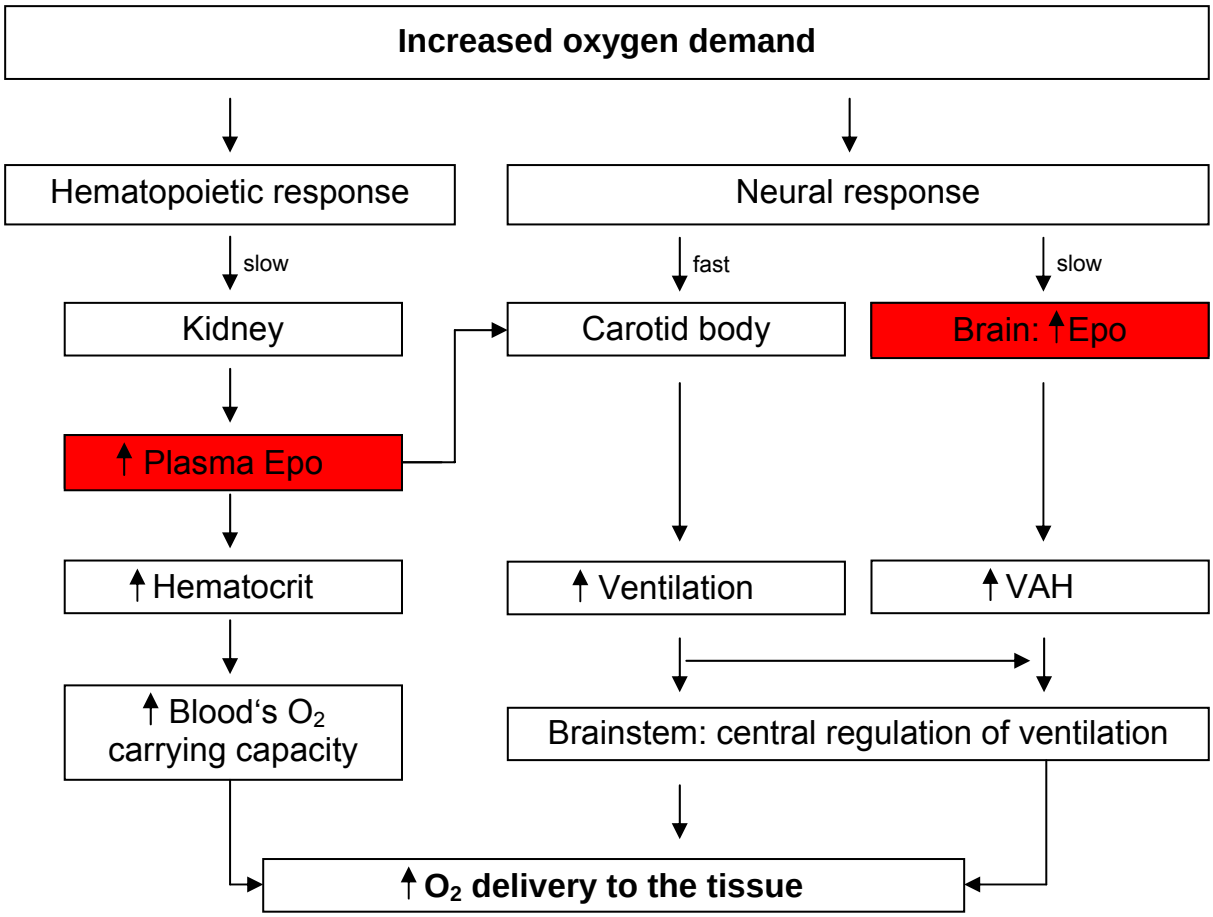

Figure 1. Model of ventilator acclimatization to hypoxia (VAH) showing the contribution of cerebral and plasma Epo.
In the first minutes of hypoxia, carotid bodies transmit the information of the blood's reduced PaO_2 to the respiratory areas in the brainstem resulting in a quickly increased ventilation. Maintained hypoxia activates long-term regulatory mechanisms. After a few hours of hypoxic exposure Epo synthesis in brain and kidney is initiated in mice. Plasma Epo is upregulated in order to augment red blood cell number to allow an augmented O_2 carrying capacity and also to contribute to the regulation of ventilation by binding to carotid body glomus cells (adapted from Soliz, Gassmann et al. 2007).

3.1. Ventilation

In unicellular organisms, simple diffusion is sufficient for gas exchange. Every cell is constantly bathed in the external environment, with only short distance for gases to diffuse. In contrast, complex multicellular animals such as mammals have a much greater distance between the environment and their innermost cells, thus, a respiratory system is needed for effective gas exchange. The process of oxygen exchange between the outside air and the cells, and the transport of carbon dioxide

(CO_2) from the cells out of the organism is called respiration. In air-breathing vertebrates respiration of oxygen includes three stages:

- Ventilation: gas exchange between lungs and environment. As this is the main topic of this work, the ventilatory process is explained below in more detail.
- Pulmonary gas exchange: oxygen diffusion from lungs to red blood cells through the alveolo-capillary membrane.
- Oxygen transport within the organism and delivery to the tissues.

The constant renewal of air in the alveolus guarantees efficient O_2 supply and CO_2 elimination as metabolic waste. The pulmonary circulation receives the total output of the right heart ventricle, perfuses the alveolar capillaries, and participates in gas exchange via passive diffusion of these gases between the alveolus and pulmonary capillaries. The oxygen in the plasma is taken up by the red blood cell's hemoglobin and is transported to the tissue. The increase of the CO_2 concentration at tissue level causes a progressive acidification that triggers the "Bohr effect" that enhances tissue oxygenation. In the cell oxygen participates in the oxidative phosphorylation process for the adenosine triphosphate (ATP) production in the mitochondria (Guyton 2000).
As mentioned before, ventilation is defined as the gas exchange between the environment and the lungs. The total volume of air breathed per minute (minute ventilation) is determined by the tidal volume (TV, volume of each breath) and the respiratory frequency (fR, number of breaths per minute). The increase in minute ventilation that occurs upon increased metabolic rate or environmental hypoxia can be mediated by an increase of VT, fR, or both. In 1997, Ganong and coworkers showed that mammalian lungs can increase the ventilatory performance 20 fold over the resting ventilation (Ganong 1997). This outstanding property allows human to live permanently at altitudes higher than 4000m, such as populations native to the Tibetan and Andean Plateau (Beall 2007).

3.1.1. Regulation of ventilation in hypoxic environment

Increased ventilation is the first response to acute reduction of environmental oxygen (hypoxia). This fast reaction is mediated through the interaction of two systems:

- Arterial chemoreceptors in the peripheral nervous system.
- Respiratory network in the brainstem.

3.1.1.1. Arterial chemoreceptors: Carotid bodies

Oxygen partial pressure (PaO_2) can be sensed by all mammalian cells. However, the chemoreceptors in the carotid artery are organs specialized in the transmission of the information of reduced oxygen content in the blood flowing to the brain (Kumar 2007; Ward 2008). Since the first description of the carotid bodies as sensory receptors by De Castro 1928, numerous researchers were and still are investigating the function of carotid bodies (DeCastro 1928). Carotid bodies are located near to the bifurcation of the carotid artery. While rat carotid bodies are well recognizable organs (Fig. 2), carotid bodies of mice are multilobulated structures.

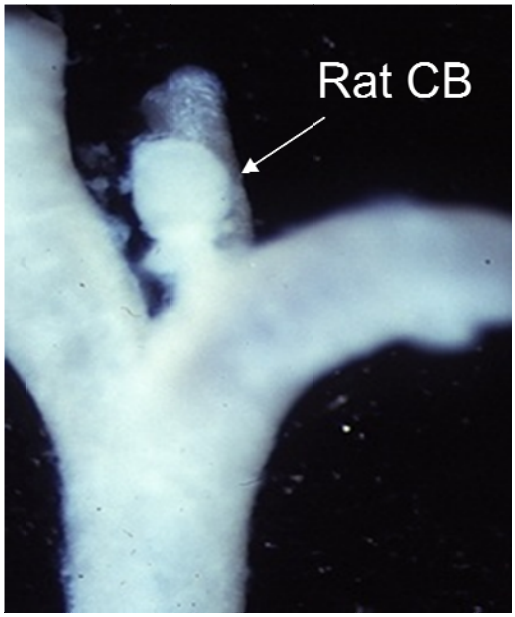

Figure 2. Rat carotid bifurcation with carotid body (CB).

Carotid bodies represent the body's most vascularized organs (Gonzalez, Almaraz et al. 1994). They are highly innervated, for example by the carotid sinus nerve, a branch of the glossopharyngeal nerve that is responsible for this organ's main innervation. The carotid bodies also receive sympathetic and parasympathetic efferent innervation (Fidone, Gonzalez et al. 1988; Gonzalez, Almaraz et al. 1994). The function of the carotid bodies is to measure the chemical changes in the freshly oxygenated blood that returns from the lung-circulation and is pumped by the heart to the brain via the carotid artery. Thus, the main stimulus for carotid bodies is the PaO_2 decline, but carotid bodies are also stimulated by changes in pH, temperature, arterial flow and pressure, osmolarity or hypoglycemia (Fidone, Gonzalez et al. 1988; Lopez-Barneo, Pardal et al. 2001; Pardal and Lopez-Barneo 2002). Carotid bodies are formed by clusters of mainly two types of cells that are surrounded by a dense network of capillaries:

- Type I cells: sensory elements of the carotid body
- Type II cells: supporting cells

Type I cells, also called glomus cells, are chemosensitive cells from neuronal origin. Under hypoxic stimulus, oxygen-sensitive potassium-channels (K^+) in the membrane of glomus cells close thereby leading to a membrane depolarization (Fig. 3) (Peers and Buckler 1995; Lopez-Barneo, Pardal et al. 2001; Lopez-Barneo 2003; Weir, Lopez-Barneo et al. 2005). In turn this depolarization activates voltage-gated calcium (Ca^{2+}) channels. The increase of cytosolic Ca^{2+} concentration triggers the release of granules containing neurotransmitters or neuromodulators (Oomori, Nakaya et al. 1994; Nurse and Zhang 1999; Prabhakar 2000; Zhang, Zhong et al. 2000). Thus, the activated afferent fibres send the information to the central nervous system that causes corrective changes in ventilation to maintain blood gas and pH homeostasis.

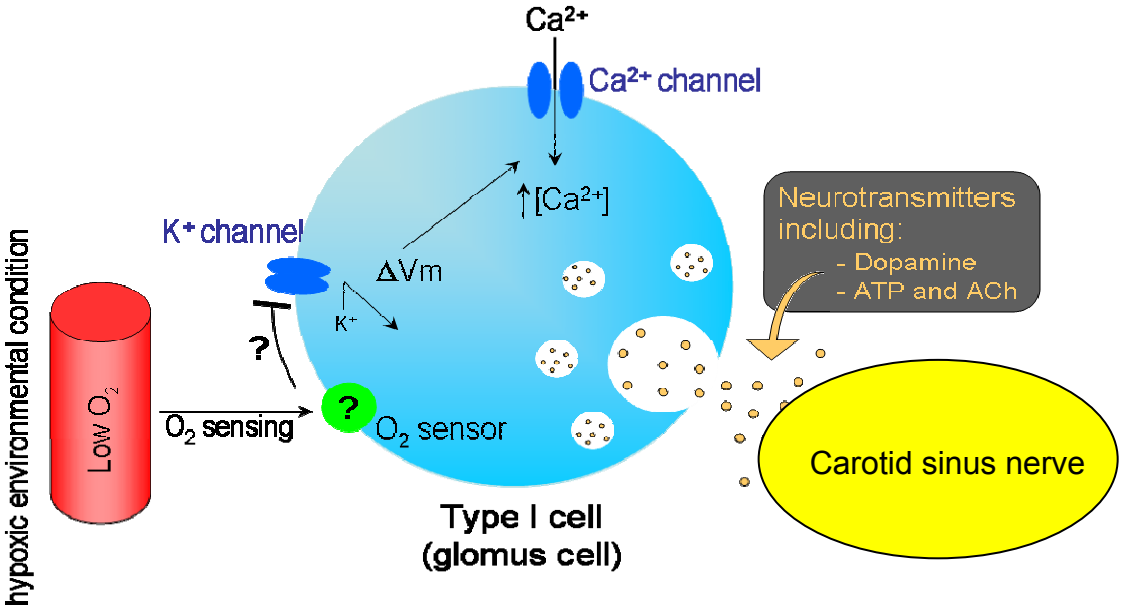

Figure 3. Chemosensory pathway of signal transmission from glomus cells to the carotid sinus nerve.

Acethylcholine and ATP seem to be the principal neurotransmitters at the nerve endings of type I carotid body cells (Zhang, Zhong et al. 2000; Rong, Gourine et al. 2003). Catecholamines, mostly dopamine, are considered to be potent negative modulators of the response to hypoxia of the carotid body (Finley and Katz 1992; Gonzalez, Vicario et al. 1995).

Type II cells, or sustentacular cells, surround the glomus cells. Type II cells resemble glia and act as supporting cells (Kondo, Iwanaga et al. 1982). In case of a decreased oxygen supply, occurring for example at high altitude, carotid bodies are the first organs that respond to this new environmental condition (Lopez-Barneo, Pardal et al. 2001; Peers and Kemp 2001). This fast reaction makes carotid bodies crucial for the survival of acute and chronic hypoxia.

3.1.1.2. Respiratory areas in the brainstem

The largest concentration of respiratory premotor neurons, the ventral respiratory group (VRG), is located in the ventrolateral medulla of the brain. The VRG forms a long rostrocaudal column that touches rostral to a compact cell cluster, the Bötzinger complex. It is thought that the Bötzinger complex is a principal source of reciprocal

inhibition in the respiratory network. Cells of the Bötzinger complex have widespread projections through the VRG and the ventrolateral nucleus of the tractus solitarius (NTS) that also contains respiratory neurons. Between the VRG and the Bötzinger complex, another cluster of respiratory neurons is located, the so-called Pre-Bötzinger complex (preBötC), that is the kernel of respiratory rhythmogenesis. The elimination of the preBötC abolishes rhythmicity (Smith, Ellenberger et al. 1991). Several nuclei outside of the VRG and the NTS project to motor neurons or regions that contain respiratory bulbospinal neurons (Fig. 4).

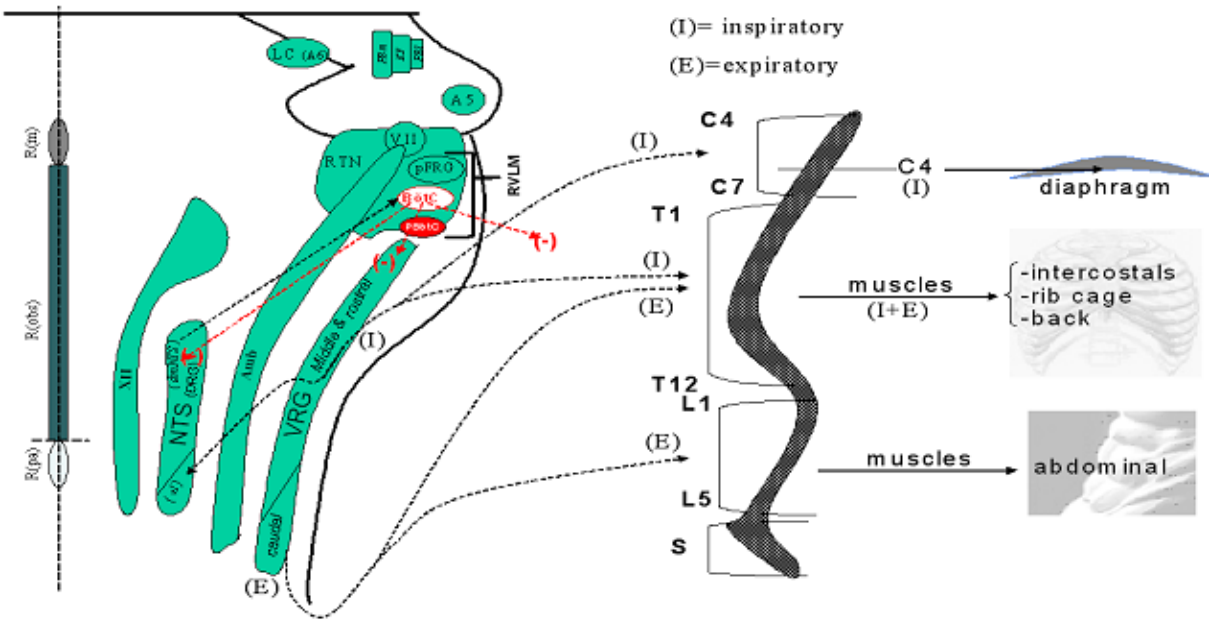

Figure 4. Respiratory areas in the brainstem and their connection to the spinal cord and corresponding respiratory muscles.
Horizontal cut of the brainstem. Ventral respiratory group (VRG), nucleus tractus solitarius (NTS), rostroventrolateral medulla (RVLM), parafacial respiratory group (pFRG), facial nerve (VII), retrotrapezoidal nucleus (RTN), nucleus ambiguus (Amb), pallidus, obscurus and magnus raphe (Rpa, Robs, Rm), glossofaryngeal nerve (XII), koliker fuse (KF), parabrachialis (PB), caudal (C), thoracic (T), lumbar (L) and sacral (S) vertebrae (adapted from Soliz, 2006).

3.2. Hypoxic ventilatory response (HVR)

At sea level, where the standard atmospheric pressure is 760mmHg, air has a PaO_2 of 159mmHg. With the increasing altitude, the atmospheric pressure decreases exponentially, leading to a parallel reduction of PaO_2, thus a decreased availability of oxygen in the air. In the case of acute environmental hypoxia, carotid bodies immediately sense the changed conditions in the arterial blood. This information is forwarded through the carotid sinus nerve to the NTS that acts as an amplifier and activates neurons of the ventral and the dorsal respiratory groups in the brainstem (Fig. 5). This activation leads to an increase of ventilation to ensure an optimal oxygen supply. The difference between the hypoxic hyperventilation and the normoxic ventilation is termed hypoxic ventilatory response (HVR). The HVR reflects the reaction of the neurological system that integrates the hypoxic stimulation of carotid bodies, the central translation of peripheral inputs to the phrenic nerve and the metabolic response of the organism (Prabhakar and Kline 2002).

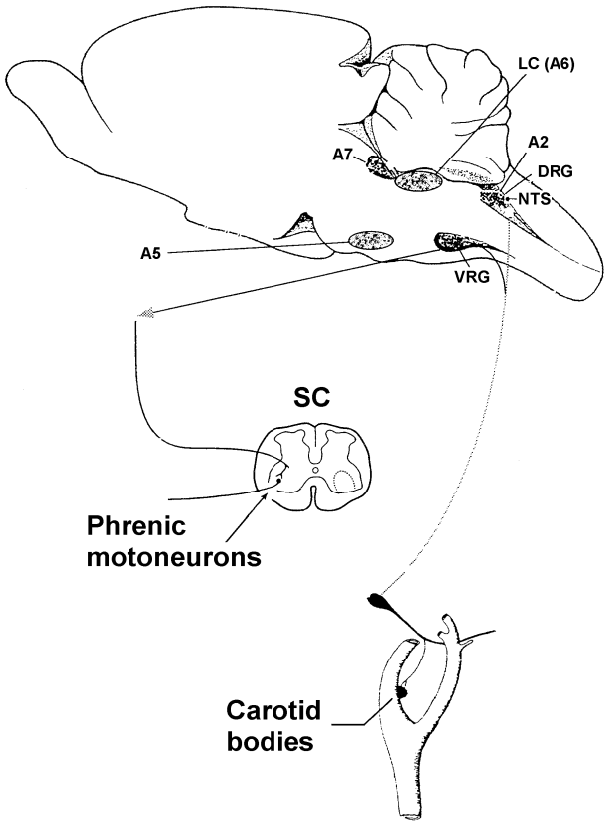

Figure 5. Schematic representation of the respiratory pathway under hypoxic conditions.
VRG: ventral respiratory group; DRG: dorsal respiratory group; NTS: nucleus tractus solitarious; SC: spinal cord; LC, locus coeruleus. A2, A5, A6, A7: catecholaminergic cell groups in the brainstem (Adapted from Soliz, 2006).

3.3. Ventilatory acclimatization to chronic hypoxia (VAH)

Depending on the duration of exposure to hypoxia, different changes in ventilation can be observed (Fig. 6).

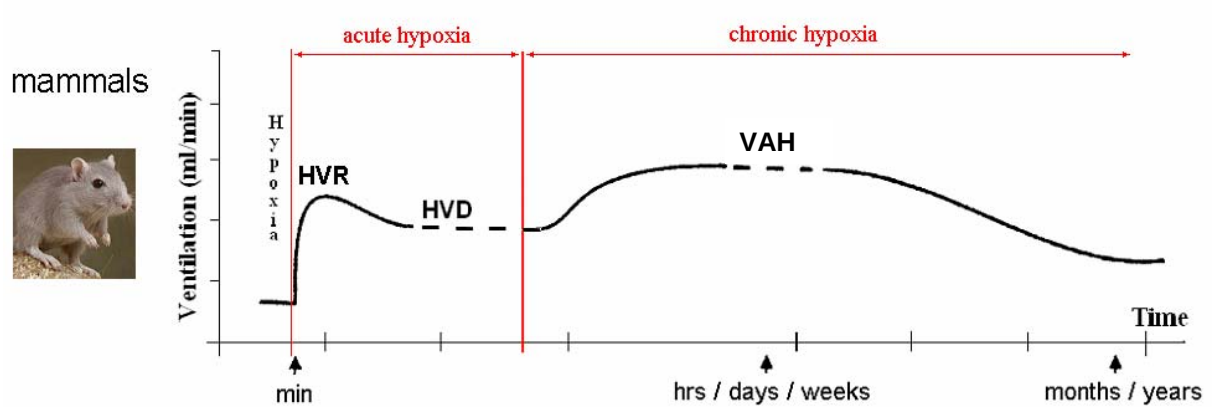

Figure 6. Schematic representation of HVR in dependency of time
Theory of ventilatory acclimatization to hypoxia (Powell, Milsom et al. 1998): VE: ventilation, HVR: hypoxic ventilatory response, HVD: hypoxic ventilatory depression, VAH: ventilatory acclimatization to hypoxia

As an acute response to a hypoxic environment mammals show a hyperventilation that is mediated by the carotid bodies (HVR). In this situation, hyperventilation is needed to maintain an optimal oxygen supply. After some minutes, a second augmentation of the ventilation occurs that is termed short term potentiation (Millhorn and Eldridge 1986). When the hypoxic stimulus lasts for more than 10-30 minutes, ventilation is depressed. This phase is called hypoxic ventilatory depression (HVD). For long time it was thought that HVD occurs due to the respiratory alkalosis occurring during hyperventilation. However, Bisgard and co-workers showed in 1995 that HVD also occurs under isocapnic hypoxia, suggesting that HVD occurrence is due to central ventilatory inhibition (Bisgard 1995). It is hypothesized that the physiological function of HVD is a conservation of energy during hypoxia (Neubauer, Melton et al. 1990).

After days or weeks in hypoxia, ventilatory acclimatization (VAH) occurs. VAH is a time dependent increase in ventilation, until reaching a plateau, with the goal to augment tissue oxygenation. The time in which VAH appears is species dependent. VAH develops in humans after 10 days (Dempsey and Forster 1982; Bisgard 1995), in rats after 7 days (Olson and Dempsey 1978; Dempsey and Forster 1982; Aaron

and Powell 1993), in cats after 2 days (Tatsumi, Pickett et al. 1995) and in mice after 3 days (Malik, Peng et al. 2005).
Several publications showed that carotid bodies are crucial for VAH (Busch, Bisgard et al. 1985; Smith, Bisgard et al. 1986). Note that the acclimatization to hypoxia is also influenced by several other factors. As there is a sexual dimorphism in VAH, female sexual hormones play most probably an important role in ventilation (see below).

3.4. Molecular adaptation to hypoxia

The up-regulation of gene expression is mediated mainly by the activity of the hypoxia-inducible factor-1 (HIF-1). HIF-1 protein is degraded by oxygen. HIFs regulate the transcription of more than 70 genes that are involved in cellular processes that act against the decreased oxygen availability by decreasing oxygen dependence and consumption of the cells, and by increasing the efficiency of the oxygen delivery to the cells (Lisy and Peet 2008). The best known molecule upregulated by HIF-1 and HIF-2 is Epo (Siren and Ehrenreich 2001; Jelkmann 2007).

3.5. Erythropoietin

Epo is a glycoprotein that is primarily produced in the kidney upon hypoxic stimulation (Jacobson, Goldwasser et al. 1957; Jelkmann 1992). For fetal synthesis, in contrast, the liver is the main production site for Epo (Lucarelli, Porcellini et al. 1968). Flake showed in 1987 that a fetal lamb liver that was transplanted in an adult sheep produced significantly more Epo than a transplanted newborn liver. (Flake, Harrison et al. 1987) This finding indicated that after birth either the Epo-producing capacity of the liver or its sensitivity to hypoxia decreases.
Nowadays it is known that Epo is not only expressed by the kidney and the liver. Sasaki and co-workers reported the presence of the Epo receptor (EpoR) in rat pheochromozytoma PC12cells, that are considered as a reliable model for neuronal cells (Sasaki, Masuda et al. 2001). Different groups including ours showed later that Epo and its receptor are as well expressed by several other tissues including brain (Digicaylioglu, Bichet et al. 1995; Marti, Wenger et al. 1996; Marti, Gassmann et al.

1997), lung (Tan, Eckardt et al. 1992), retina (Grimm, Wenzel et al. 2002) and testis (Magnanti, Gandini et al. 2001).

3.5.1. Erythropoietin has several functions

Epo plays a key role in erythropoiesis (Jacobson, Goldwasser et al. 1957; Jelkmann 1992) as it maintains production of red blood cells by inhibiting apoptosis of erythroid progenitor cells and by stimulating their proliferation and differentiation into normoblasts (Koury and Bondurant 1990; Silva, Grillot et al. 1996). Under hypoxic conditions, the increased expression of Epo by the kidney causes a higher amount of red blood cells that lead to an improved oxygen carrying capacity of the arterial blood. (Gassmann, Heinicke et al. 2003; Sasaki 2003)

With the finding that Epo is expressed in brain and the fact that the blood-brain-barrier excluded a systemic function of Epo, it was obvious that Epo must have a local cerebral function. In 1998 it has been reported for the first time that Epo protects neurons from ischemic damage in vivo (Sakanaka, Wen et al. 1998). This neuroprotective effect of Epo under pathological conditions was confirmed by several other studies (Bernaudin, Marti et al. 1999; Brines, Ghezzi et al. 2000; Calapai, Marciano et al. 2000; Marti, Bernaudin et al. 2000; Wiessner, Allegrini et al. 2001). In 2002 the clinical benefit of Epo in the therapy of stroke patients was published for the first time (Ehrenreich, Hasselblatt et al. 2002). Recently, our laboratory showed that apart from the implications under pathological situations, cerebral Epo also has a physiological function: when exposed to a hypoxic environment, Epo affects the neural respiratory network by increasing hypoxic minute ventilation (Soliz, Joseph et al. 2005).

3.5.2. Impact of Epo on hypoxic ventilation

To show the effect of Epo on hypoxic ventilation, experiments with three different animal models were performed. The first model was a transgenic mouse line, Tg21, that constitutively overexpresses human Epo in brain only (fourfold over wildtype animals (WT)), but shows normal Epo plasma levels and is as such the perfect model for studying the influence of neural Epo on the hypoxic ventilation (Wiessner, Allegrini et al. 2001). As second model WT animals were used that were injected via the tail

vein with recombinant human Epo (rhEpo) prior to the respiratory measurements. This model allowed us to learn about the acute influence of plasma Epo on hypoxic ventilation. Finally, as third model, another transgenic mouse line termed Tg6 was used. Tg6 mice show elevated Epo levels in plasma (12 fold over WT, due to a constitutive overexpression of human Epo in lung tissue) and in brain (26 fold over WT). The elevated Epo plasma levels lead to excessive erythrocytosis with hematocrit values up to 90% (Ruschitzka, Wenger et al. 2000; Wagner, Katschinski et al. 2001; Heinicke, Baum et al. 2006). These high hematocrit values result in an increased blood volume (Ruschitzka, Wenger et al. 2000; Vogel, Kiessling et al. 2003) and also in elevated central venous and pulmonary artery pressures (Wagner, Katschinski et al. 2001). Surprisingly, Tg6 mice show no hypertension, a normal cardiac output and a normal heart rate (Ruschitzka, Wenger et al. 2000; Wagner, Katschinski et al. 2001; Vogel, Kiessling et al. 2003). As adaptive mechanism to the excessive erythrocytosis Tg6 mice show an increased expression of the endothelial nitric oxide synthase (eNOS) resulting in an elevated synthesis of nitric oxide (NO) (Ruschitzka, Wenger et al. 2000; Hasegawa, Wagner et al. 2004). It has been reported that survival of Tg6 mice depends on NO bioavailability (Ruschitzka, Wenger et al. 2000; Hasegawa, Wagner et al. 2004) due to the NO induced vasodilatation. As another adaptive mechanism Tg6 mice regulate blood viscosity by improving red blood cell deformability (Vogel, Kiessling et al. 2003).

Taking together the analyses of ventilation of these three models it was concluded that Epo regulates hypoxic ventilation in mice by interacting with brainstem and carotid bodies (Soliz, Joseph et al. 2005; Heinicke, Baum et al. 2006).

3.6. Sexual dimorphism in the control of hypoxic ventilation

Recent reports show that the physiological response of humans and mammals to hypoxia is gender dependent. Ovarian steroids are known since long as potent stimulants of the peripheral and central respiratory system (Bayliss, Millhorn et al. 1987; Bayliss, Cidlowski et al. 1990; Hannhart, Pickett et al. 1990; Tatsumi, Pickett et al. 1997). Indeed, several studies reported that women and female rats have a better capacity to adapt to hypoxia (Kryger, McCullough et al. 1978; Joseph, Soliz et al. 2000; Joseph, Soliz et al. 2002). In line with these findings, it was reported that women are less susceptible to a number of hypoxia-associated syndromes and

sickness in infancy and adulthood, such as sudden infant death syndrome (Vance, Boyle et al. 2002; Mage and Donner 2004; Mage and Donner 2006) and sleep apneas (Dursunoglu, Dursunoglu et al. 2006; Dahlqvist, Dahlqvist et al. 2007; Valipour, Lothaller et al. 2007).

In view that female mammals cope better with hypoxia and considering that cerebral and systemic Epo is a key factor controlling the hypoxic ventilatory response by interacting with the brainstem and the carotid bodies, we expected that Tg6 female mice will show elevated ventilation and enhanced capacity to adapt to chronic hypoxia compared to WT female animals and male transgenic mice. Once again, Tg6 mice constitutively overexpress Epo in brain and lung tissue, the latter expression leading to 12 fold elevated Epo plasma levels.

Indeed, respiratory measurements reported that female Tg6 mice show a significantly higher ventilation compared to WT females and Tg6 males in normoxia (21%O_2) as well as in acute moderate (10%O_2) and severe (6%O_2) hypoxic conditions (Soliz, Thomsen et al. 2009). However, when these experiments were repeated using mice preexposed to chronic hypoxia (10%O_2 for 3 days) unexpected results were obtained: while WT females acclimatized, Tg6 females did not show any acclimatization behaviour upon preexposure to chronic hypoxia.

3.7. Aim of the project

As it was reported that Epo influences hypoxic ventilation and it is known that women and also female mice cope better with hypoxic conditions than males, we wanted to investigate the ventilatory acclimatization to chronic hypoxia in Tg6 females, that show elevated Epo levels in plasma and brain. As we observed that Tg6 females are not able to acclimatize to chronic hypoxia while Tg6 males show no problems to do so, the aim of the present study was to find out whether Epo is interacting with female sexual steroids causing this blunted VAH and whether this interaction occurs on central or peripheral level of the control of hypoxic ventilation.

4. MATERIAL AND METHODS

4.1. Transgenic mice

The Epo-overexpressing transgenic mouse lines were generated by pronuclear injection of human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B chain promoter into fertilized oocytes derived from B6C3 hybrid mice (Ruschitzka, Wenger et al. 2000). One resulting mouse line TgN(PDGFBEPO)321ZbZ (Tg6) that showed increased Epo levels in plasma and in brain, was backcrossed to C57BL/6 mice by mating hemizygous males to WT C57BL/6 females. Half of the offspring was hemizygous for the transgene, while the other half was wildtype and as such was used as control. The second resulting transgenic mouse line TgN(PDGFBEPO)322ZbZ (Tg21) that showed increased Epo levels in brain only was bred to homozygosity in a C57Bl/6 background (Wiessner, Allegrini et al. 2001; Soliz, Joseph et al. 2005). All experiments were performed in three to five month old female mice. Animal experimentations were performed in accordance with the Swiss animal protection laws and institutional guidelines.

4.2. Exposure to chronic hypoxia

Mice were placed 72 hours prior to the respiratory measurements in an Invivo2 1000 hypoxic workstation (Ruskinn, UK) with free access to food and water. The oxygen content of the workstation was gradually decreased from room air to 10%O_2 within one hour. Chronic hypoxic exposure was performed during three days. (Kline, Peng et al. 2002; Bin-Jaliah, Maskell et al. 2004; Malik, Peng et al. 2005; Jacono, Peng et al. 2006) Subsequently mice were returned to room air and placed in the plethysmograph for respiration measurements.

4.3. Ventilatory measurements

Respiration was monitored by a whole-body flow-though plethysmograph (EMKA Technologies, France) as described (Soliz, Joseph et al. 2005). Briefly, after body weight measurement, mice were placed in a 600 ml chamber continuously supplied

with airflow at 0.7-0.8l min^{-1} using flow restrictors. Ventilation (VE) was calculated as the product of tidal volume (VT) and respiratory frequency (fR) and normalized to 100 g of body weight. After a period of acclimatization to the chamber (about 1h) baseline ventilation was measured (normoxia, 21% O_2). Hypoxia was achieved by flushing air balanced in N_2 using a gas-mixing pump (Digamix, type M302 a-F, H Wösthoff e.H.G., Germany). The inspired oxygen fraction (FIO_2) was gradually decreased from 21% to 10% O_2 over three minutes. Respiration at 10% O_2 was measured for 20 minutes. Then FIO_2 was further decreased to 6% over two minutes and respiration was again measured for 20 minutes.

An open-circuit system allowed measurement of O_2 consumption (VO_2, ml/min/100g; atmospheric temperature and pressure in dry air) and CO_2 production (VCO_2, ml/min/100g) during normoxia and hypoxia (10% and 6%). The fractions of O_2 and CO_2 at the inflow and the outflow of the plethysmograph chamber were measured by O_2 and CO_2 analyzers (Qubit Systems Inc., Kingston, Ontario). Body temperature in normoxia and hypoxia was measured using a rectal thermocouple (Fluke Corporation, USA) under all conditions following the hypoxia protocol above.

For sensitivity measurement of the carotid body type 1 cells to changes in the PaO_2 in the arterial blood, animals were anaesthetized to avoid any movement during the brief exposure to 100%O_2 (Dejours test). Two minutes after the i.p. injection of urethane solution (1.2g/kg), that has only a minimal effect on respiratory frequency and cardiac dynamics (Kline, Peng et al. 2002; Bin-Jaliah, Maskell et al. 2004; Jacono, Peng et al. 2006) mice showed regular ventilation and a normal respiratory frequency. Baseline respiration was measured at 21% O_2 for 20 seconds. The chamber was then suddenly flushed with 100% O_2 and the decline of respiration was recorded for one minute. The magnitude of the transient ventilatory decline was calculated as the difference between baseline and hyperoxic respiration parameters.

4.4. Ovariectomy

Tg6 and WT females were ovarectomized at three months. In brief, anaesthesia was induced with a gas mixture (4% halothane, 70% N_2O and O_2) and maintained by reducing the inspired halothane to 1-1.5%. Body temperature was maintained by a temperature-controlled heating pad. Ovaries were then removed by a bilateral dorsal incision. The wound was carefully closed and disinfected with 10% polividone iodine

(Betadine, Asta Medica, Merignac, France). Hypoxic experiments were performed after two weeks of recovery. Operated females were injected i.p. daily starting one week prior the respiratory measurement with either estradiol (0.005mg/day) or progesterone (0.1mg/day). Vehicles were treated the same but were injected with 0.1ml of 0.9% NaCl.

4.5. Carotid sinus nerve transaction (chemodenervation)

Tg6, Tg21 and WT mice were chemodenervated as described (Roux, Pequignot et al. 2000). In brief, anaesthesia was induced as described above. To prevent any functional regeneration of chemosensory fibres the carotid sinus nerves were completely removed from the cranial pole of the carotid body until reaching the branch of the glossopharyngeal nerve. Again ventilatory experiments were performed after at least two weeks of recovery from chemodenervation.

4.6. Detection of Epo binding sites and thyrosine hydroxylase

Wildtype and transgenic mice were perfused transcardially with phosphate buffer (0.1m, pH 7.4) and fixed with 4% paraformaldehyde. For the detection of Epo binding sites, animals were injected transcardially with 3000U rhEpo 15 minutes before the perfusion. After perfusion carotid bifurcations were removed and immersed in 4% paraformaldehyde for 24 hours, then cryoprotected in 30% sucrose-phosphate for 48 hours at 4°C. After freezing on dry ice carotid bifurcations were sectioned in the cryostat at 8µm. Sections were dried one hour at room temperature and then washed in phosphate-buffered saline (PBS) twice for five minutes. For blocking the tissue was exposed to 10% goat serum in TBS-T for one hour and then incubated with the first antibody against thyrosine hydroxylase at 4°C for 48 hours (NB300-173, Novus Biologicals; 1:500). After 24 hours the first antibody against rhEpo was added for 24 hours at 4°C (MAB2871, R&D Sytems; 1:100 in 3% goat serum). After two days sections were washed six times in PBS for five minutes and then incubated with the secondary antibodies for two hours at room temperature in the dark (Cy3 goat anti rabbit, Jackson ImmunoResearch, 1:200; Cy5 goat anti mouse, Jackson ImmunoResearch, 1:200). After washing three times for five minutes in PBS sections

were incubated with DAPI for five minutes (10mM, 1:5000). After the last washing step, all liquid was sucked from the plates and then plates were mounted with moviol.

4.7. Hormone measurement

Daily vaginal smears were taken to determine cycle stadium. When animals were in oestrus, Tg6 and WT females were deeply anaesthetized (4% halothane, 70% N_2O and O_2) and a blood sample was drawn by cardiac puncture for radioimmunoassay (RIA) of plasma estradiol and progesterone level (Siemens, CH)

4.8. Statistical analyses

For statistical analyses StatView software was used (Abacus Concepts, Berkeley, CA, USA). Data were analysed by two-way ANOVA. Differences were considered as significant at $p < 0.05$.

5. Results

5.1. Female mice ventilation and HVR after chronic hypoxia (3 days at 10%O_2)

Acclimatization to hypoxia leads to elevated basal ventilation compared to normoxic basal ventilation (VAH) thereby ensuring an optimal oxygen supply despite the reduction of oxygen in hypoxia. In mice this process occurs after three days (Olson and Saunders 1987; Malik, Peng et al. 2005). As expected, when we compared basal ventilation of WT and Tg6 female mice before and after VAH, we observed that WT females showed a ventilatory acclimatization due to an increase of respiratory frequency and tidal volume (Fig. 7a - c). In contrast, Tg6 females showed an impossibility to reach VAH. Basal ventilation after the exposure to chronic hypoxia (10%O_2 for 3 days) was significantly lower than in normoxia (21%O_2; Fig. 7a). This decrease was due to a reduction of the respiratory frequency (Fig. 7b) and not alterations in tidal volume (Fig. 7c).

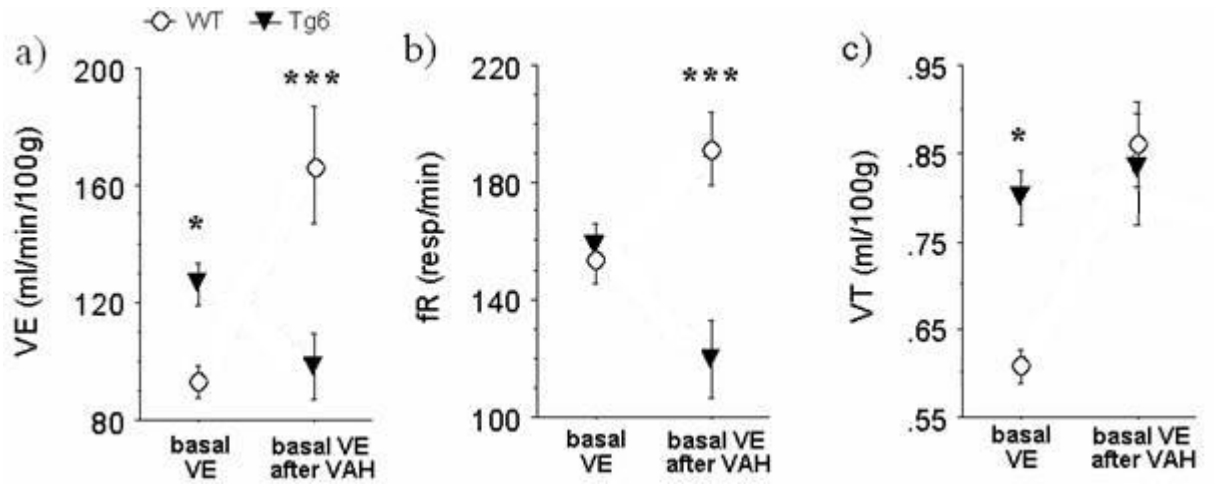

Figure 7. Blunted VAH in Tg6 females.
a) - c) measurement of basal ventilation (VE; a), respiratory frequency (fR; b) and tidal volume (VT; c) in normoxia and after three days of acclimatization to 10%O_2. *p<0.05; ***p<0.0001;
animals per group = 8.

Next we exposed females that were kept at chronic hypoxia (10%O_2) for 3 days to decreasing oxygen concentrations (10% and 6%O_2) for each 20 minutes. Again, we observed a decrease of basal ventilation in normoxia of Tg6 females compared to WT (Fig. 8a, 21%O_2). This ventilatory decrease was also observed under the conditions of moderate (10%O_2) and severe (6%O_2) hypoxia (Fig. 8a). The decreased ventilation of Tg6 females compared to corresponding WT was due to both, decreased respiratory frequency and tidal volume (Fig. 8b – c). To exclude that metabolic parameters play a role in these ventilatory changes, body temperature (Fig. 8d), oxygen consumption (Fig. 8e) and carbon dioxide production (Fig. 8f) were monitored. As there were no significant differences between WT and Tg6 we concluded that the ventilatory differences were not due to changes in the metabolism.

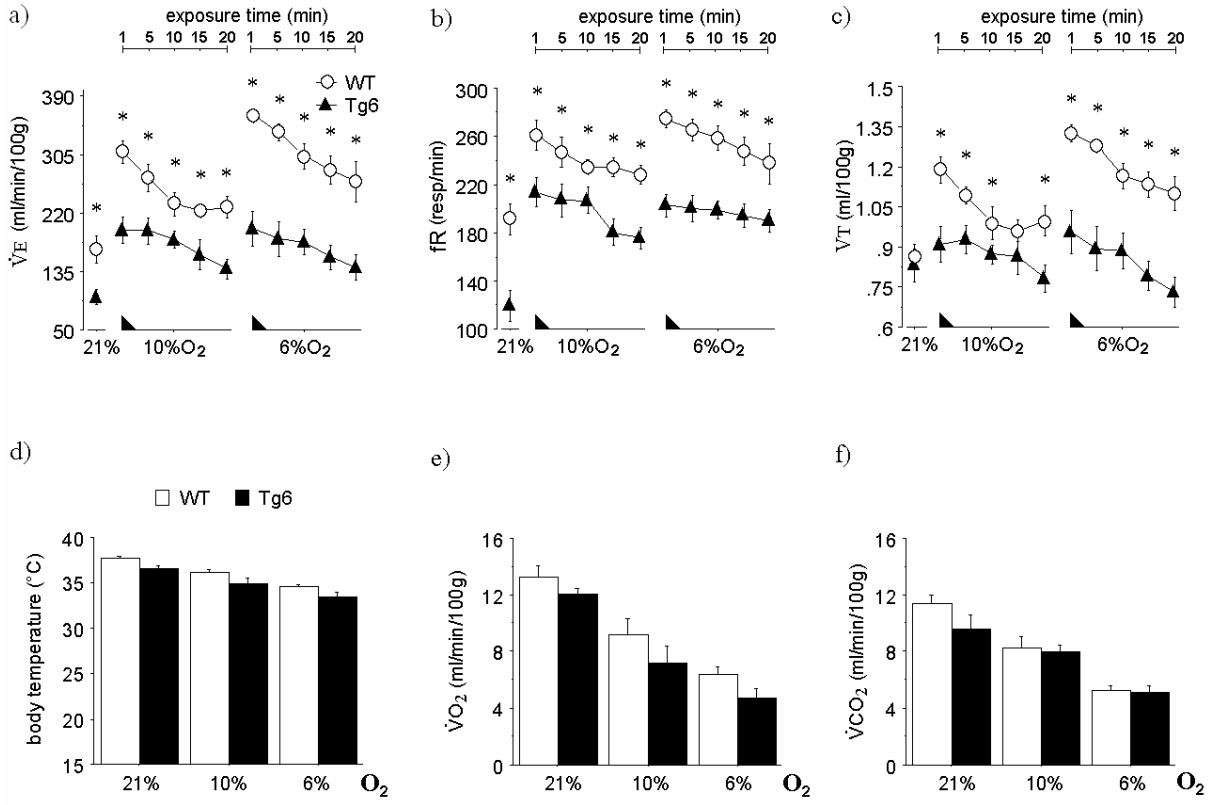

Figure 8. Ventilatory and metabolic measurements after chronic hypoxia.
a) – c) basal ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) were evaluated 1-2h after placing the animals in the plethysmograph. Hypoxia was achieved with a gradual reduction of FIO_2 from 21% to 10% O_2 (over 3min) and from 10% to 6% O_2 (over 2min). HVR was evaluated during 20 min at each 10% and 6% O_2.
d) - f), determination of body temperature (d)), O_2 consumption ($\dot{V}O_2$; e)) and CO_2 production ($\dot{V}CO_2$; f)) in WT and Tg6 female mice.
*p<0.05; animals per group = 8.

5.2. Central influence on the VAH in WT and Tg6 females

The central nervous system, especially the brainstem, controls ventilation. To examine whether the respiratory areas in the brainstem are participating in the blunted VAH response of Tg6 females, Tg6 and WT females were chemodenervated. The bilateral transection of the carotid sinus nerves abolishes the transmission of information from carotid bodies to the brainstem. Therefore, the HVR of chemodenervated animals reflects the reaction to hypoxic conditions of the brain only. When respiration of operated WT and Tg6 females were compared, we could not observe any differences, neither in ventilation, respiratory frequency nor tidal volume (Fig. 9).

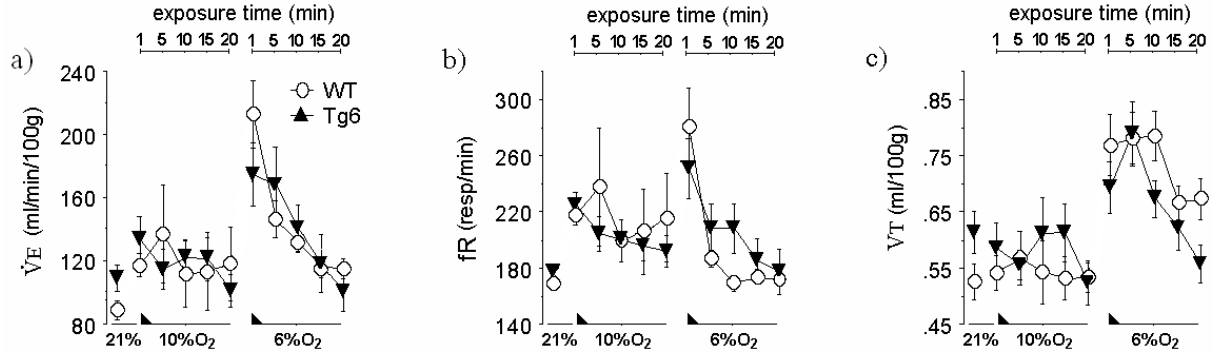

Figure 9. Minute ventilation and HVR after chronic hypoxia in chemodenervated WT and Tg6 mice.
The graphs show ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) in animals after bilateral transection of the carotid sinus nerve under conditions of normoxia, moderate (10%O2) and severe (6%O2) hypoxia. Animals per group = 8.

As the data obtained with chemodenervated Tg6 females suggested that the respiratory areas in the brainstem are not involved in the impaired VAH of Tg6 females, we performed another set of experiments using Tg21 mice. As these animals overexpress Epo in brain only, this model allowed us to test whether cerebral Epo is participating in the blunted VAH of Tg6 females, thereby omitting the influence of plasma Epo that would probably interfere with the carotid bodies (note that these animals were not chemodenervated). Again we could not find any respiratory differences (Fig. 10). In summary the results of these two experiments suggest that the central nervous system is not implicated in the ventilatory reaction of Tg6 females to chronic hypoxia.

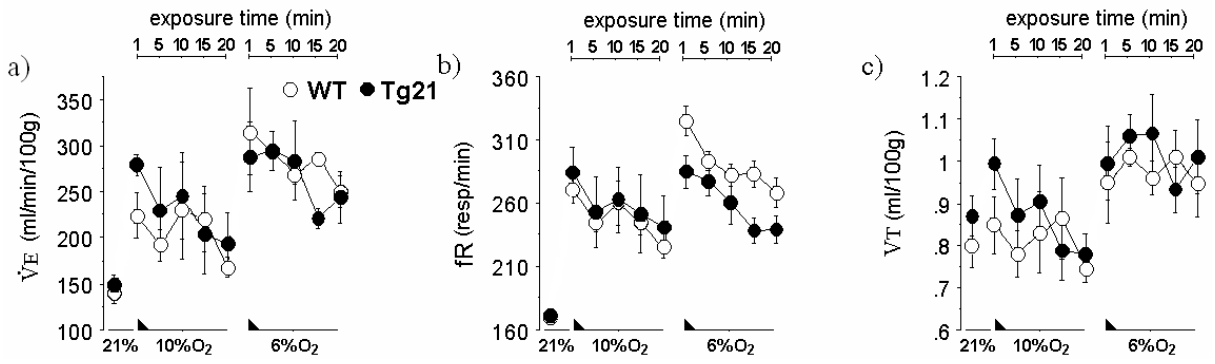

Figure 10. Minute ventilation and HVR after chronic hypoxia in WT and Tg21 mice.
The graphs show ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) in WT and Tg21 (that overexpress Epo in brain only) under conditions of normoxia, moderate (10%O2) and severe (6%O2) hypoxia. Animals per group = 6.

5.3. Epo binding sites are present in carotid body glomus cells

Carotid bodies play a key role in the establishment of VAH. As Tg6 mice have high levels of plasma Epo we postulated that Epo is influencing the VAH of Tg6 females and that this interaction occurs on the carotid body level. To determine whether Epo binding sites are present in carotid bodies we performed immunostaining in 8µm serial lateral sections from the carotid bifurcation. Animals were first injected transcardially with 3000U rhEpo. Then animals were perfused 15 minutes after the injection to give Epo time to bind to its binding sites. We expected that despite perfusion we would still find bound Epo in carotid body glomus cells due to the high dose of rhEpo that was injected. We performed doublestaining for Epo and thyrosine hydroxylase (TH) that is a reliable marker to identify carotid body glomus cells. The staining showed an overlap of TH and Epo, thus, we conclude that Epo binding sites are present in carotid bodies (Fig. 11).

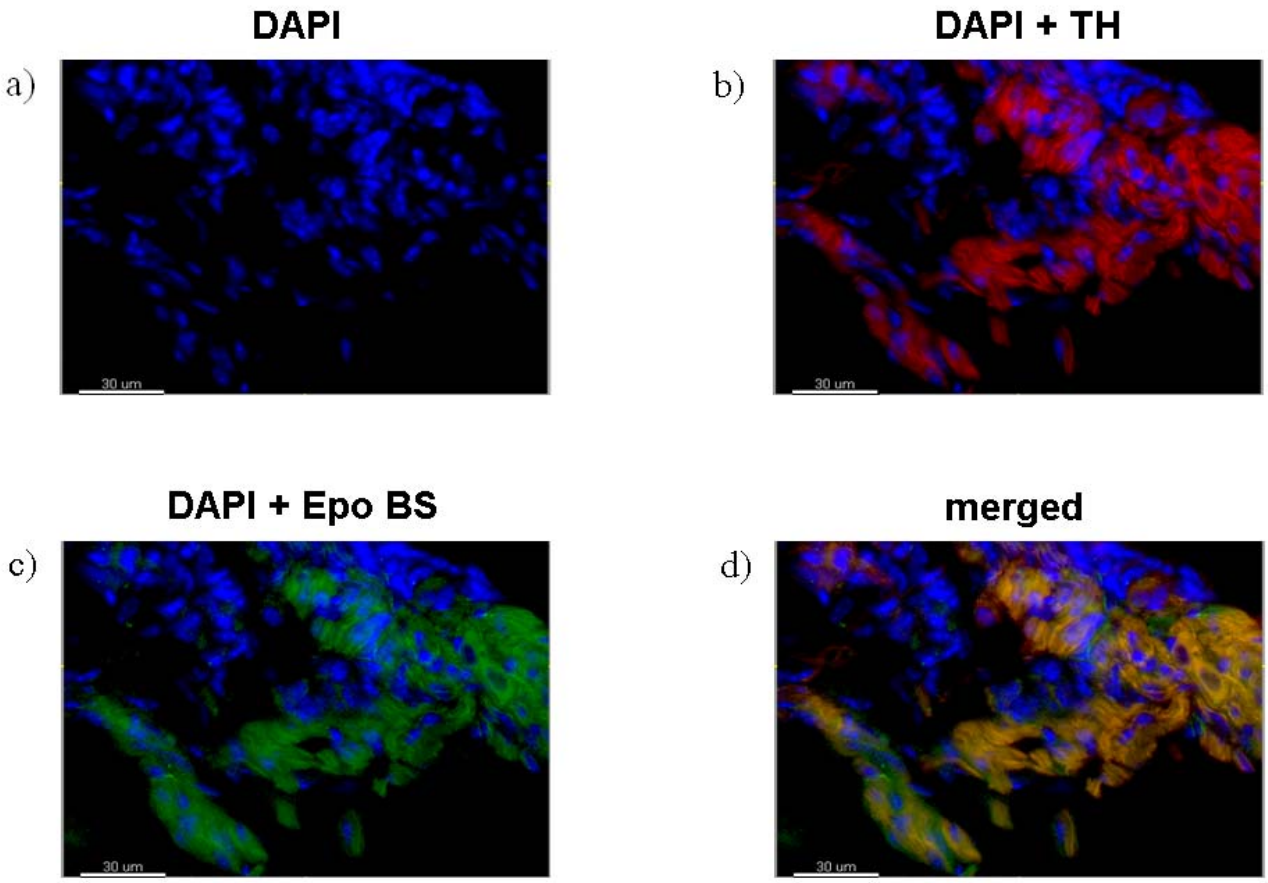

Figure 11. Epo binding sites in the carotid body.
Nuclei were made visible by dapi (a). Identification of carotid body cells in the carotid bifurcation was achieved by immunodetection of thyrosine hydroxylase (TH; b). Identification of Epo binding sites was achieved by immunodetection of bound rhEpo (c) for showing a co-localization of TH and Epo binding sites (d).

5.4. Minute ventilation and HVR in ovarectomized WT and Tg6 mice

As the blunted VAH was only reported in Tg6 females and not in Tg6 males (Soliz, Soulage et al. 2007) nor in WT females we made the suggestion that female sexual steroids (estradiol, progesterone) interact with Epo at the carotid body level. To this end we first measured the plasma levels of both hormones in WT and in Tg6 females. Our results showed that while there were no differences in the concentration of progesterone, Tg6 females showed a much higher estradiol concentration in the plasma compared to WT females (Fig. 12). This finding implies that there is an interaction between Epo and estradiol.

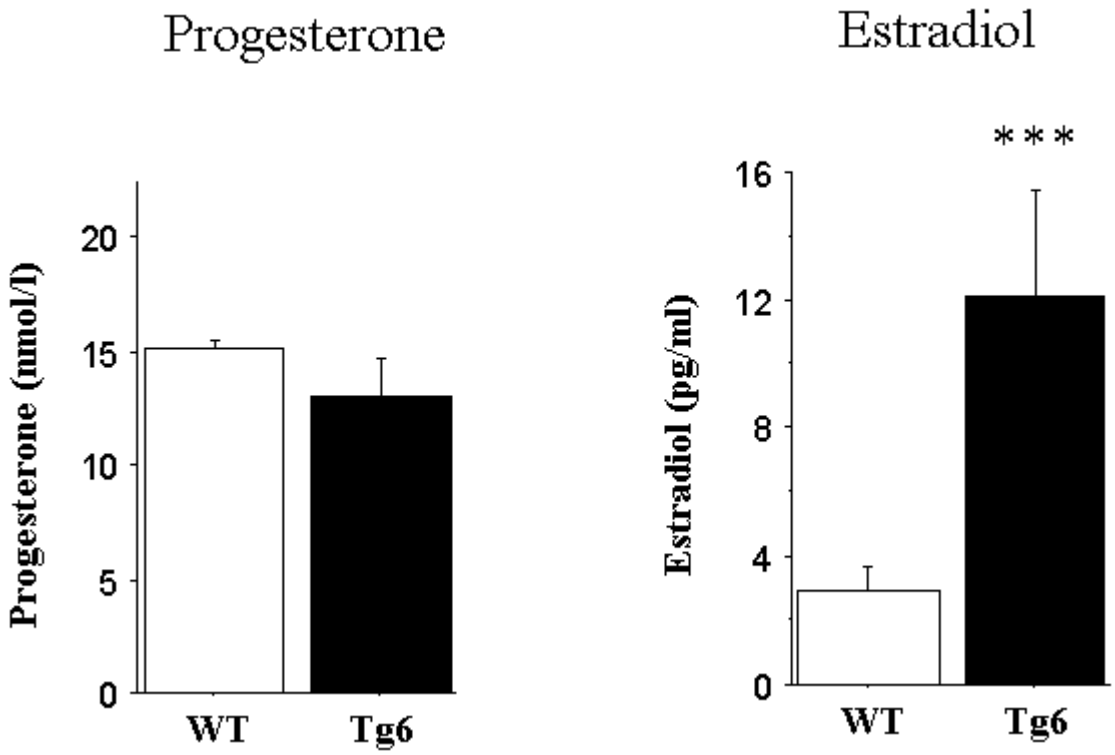

Figure 12. Sex female hormones in normoxic WT and Tg6 females.
Hormone measurement by RIA. ***p<0.0001; animals per group: 10 – 12.

Next we measured the ventilation in ovarectomized WT and Tg6 females after exposure to chronic hypoxia (10%O_2 for 3 days). As estradiol and progesterone are mainly produced in the ovaries (in nonpregnant females) ovariectomy allowed elimination of these hormones. Ovarectomized WT females showed a decreased ventilation in normoxia and moderate hypoxia (10%O_2) compared to untreated animals (Fig. 13a). This decrease was due to a lower tidal volume rather than

respiratory frequency (Fig. 13b – c). Tg6 females, in contrast, increased ventilation after ovariectomy compared to untreated animals (Fig. 13d). This recovery was due to elevated respiratory frequency and tidal volume (Fig. 13e – f). These results show that female steroids interact with Epo's ventilatory effect causing a blunted VAH response in Tg6 females.

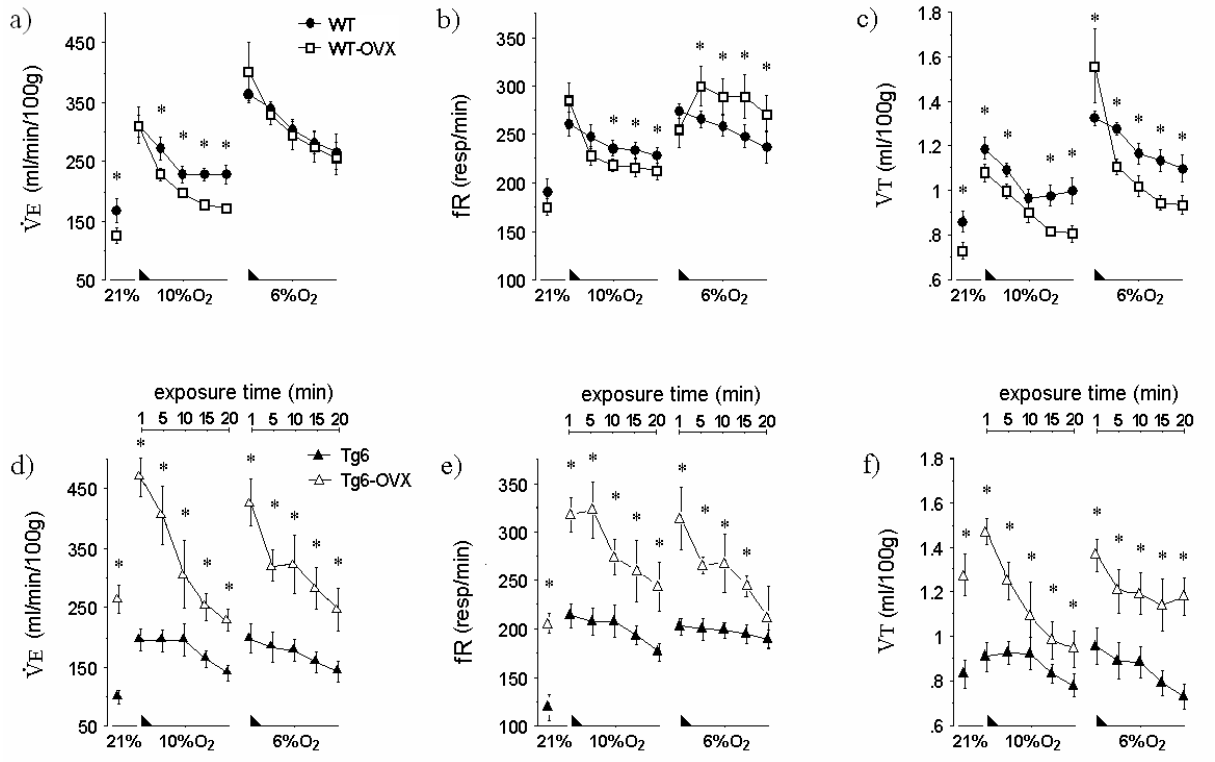

Figure 13. Minute ventilation and HVR after chronic hypoxia in ovarectomized and untreated WT and Tg6 females.
The graphs show ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) in untreated WT and ovarectomized WT under conditions of normoxia, moderate (10%O2) and severe (6%O2) hypoxia. The same conditions are shown for untreated and ovarectomized Tg6 females (d) – f)). *p<0.05; Animals per group = 8-9.

5.5. Impact of the Epo and estradiol interaction on the VAH

When estradiol was substituted (0.005mg i.p./day for 7 days) in ovarectomized WT females that were preexposed to chronic hypoxia (10%O_2 for 3days) we observed a reduced tidal volume compared to control-injected ovarectomized females (vehicles) in normoxia, moderate (10%O_2) hypoxia and the first two minutes of severe (6%O_2) hypoxia (Fig. 14c). But this decrease in tidal volume affected ventilation only in the

first two minutes of exposure to both moderate and severe hypoxia significantly (Fig. 14a). The substitution of estradiol did not have any effect on the respiratory frequency of ovarectomized WT females. In contrast the replacement of estradiol in ovarectomized Tg6 females again decreased ventilation significantly compared to control- injected females in all conditions (normoxia, moderate and severe hypoxia; Fig. 14d). This reduction was also due to a decreased tidal volume (Fig. 14f) while again there were no significant changes in the respiratory frequency (Fig. 14e). These data suggest, that estradiol in combination with Epo is responsible for the blunted VAH in Tg6 females.

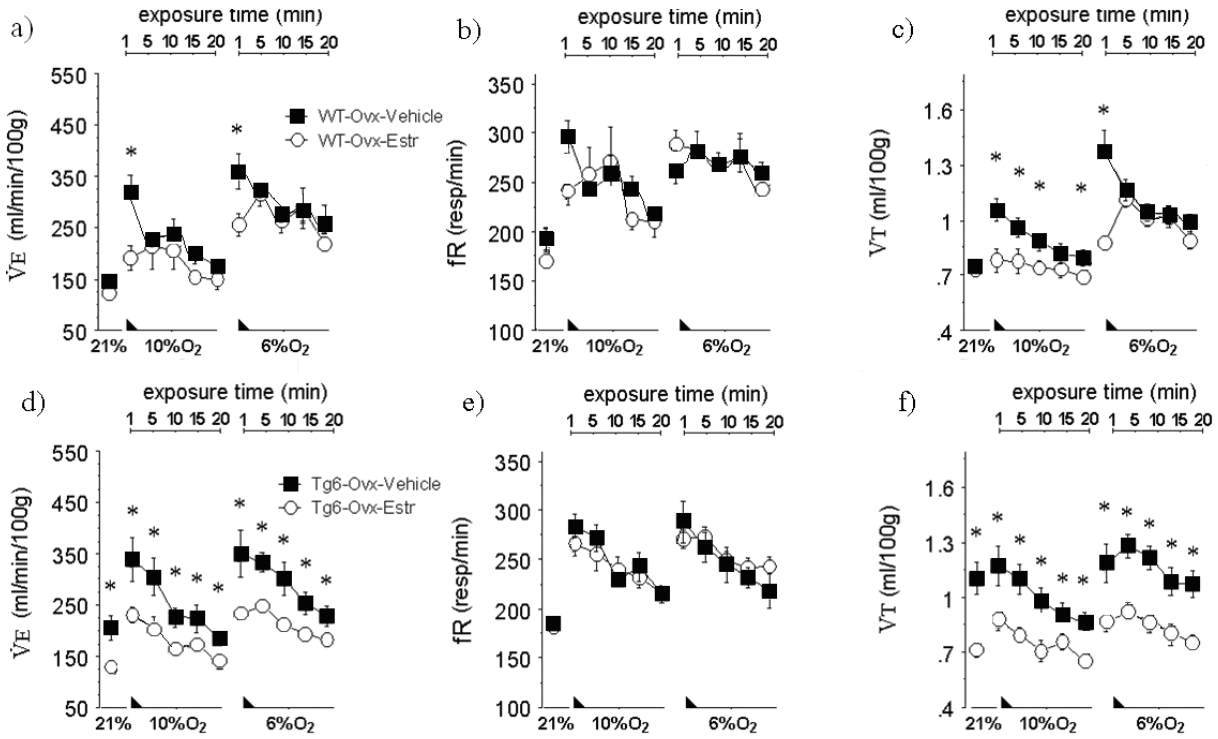

Figure 14. Minute ventilation and HVR after chronic hypoxia in ovarectomized WT and Tg6 females after substitution of estradiol.
The graphs show ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) in ovarectomized WT with NaCl injections (vehicles) and ovarectomized WT with substituted estradiol (0.005mg i.p./day for 7 days) under conditions of normoxia, moderate (10%O2) and severe (6%O2) hypoxia. The same is shown for ovarectomized Tg6 females (d) – f)). Vehicles were injected with 0.9% NaCl. *p<0.05 WT-Ovx-Vehicle vs. WT-Ovx-Estr and Tg6-Ovx-Vehicle vs. Tg6-Ovx-Estr; Animals per group = 8-9.

In a next set of experiments we examined the peripheral chemoreceptor sensitivity by applying the Dejours test after exposure of the animals to chronic hypoxia (10%O_2 for

3 days) in presence of estradiol (0.005mg i.p./day for 7 days). In this experiment animals are suddenly exposed to a brief period of hyperoxia (100%O_2) what results in a decline of ventilation. In agreement with the previous results we observed that the hyperoxia-induced ventilatory decline after substitution of estradiol was higher in ovarectomized WT females compared to ovarectomized Tg6 females (Fig. 15a). This change in WT animals compared to Tg6 mice was due to a decline in respiratory frequency (Fig. 15b) rather than tidal volume (Fig. 15c). These data imply that compared to WT Tg6 carotid bodies are less sensitive to changes in the oxygen content of the arterial blood in presence of estradiol.

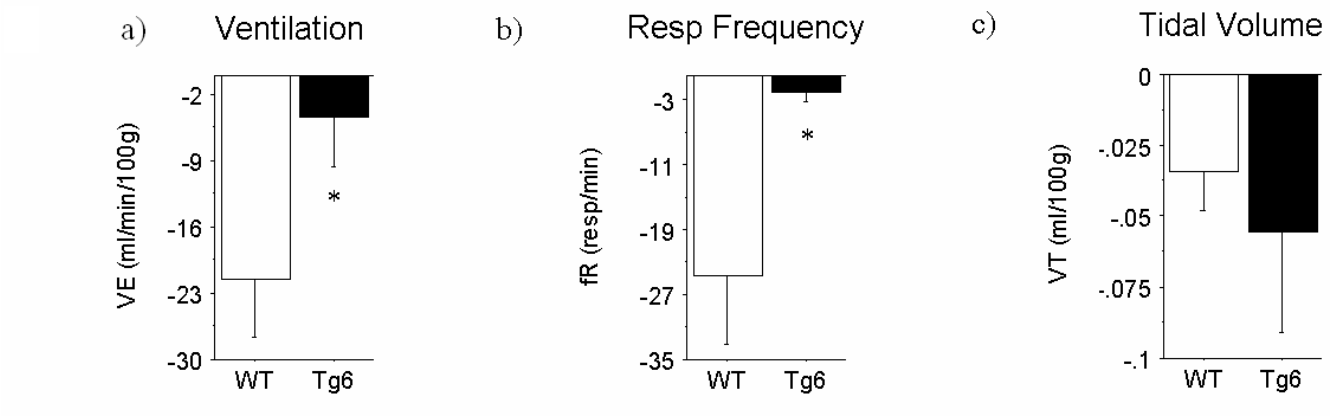

Figure 15. Dejours test after chronic hypoxia in ovarectomized WT and Tg6 females with substituted estradiol.
Decline of ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) upon transition from 21% to 100% O_2 (Dejours test). The magnitude of the transient ventilatory decline was calculated as the difference between baseline and hyperoxic respiratory parameters. The experiment was performed in urethane anaesthetized ovarectomized WT and Tg6 females after substitution of estradiol (0.005mg i.p./day for 7 days). *p<0.05; animals per group = 7-8.

5.6. Impact of the Epo and progesterone interaction on the VAH

To determine whether progesterone is also participating in the impaired VAH of Tg6 females we substituted progesterone in ovarectomized females (0.1mg i.p./ day for 7 days). The measurement of respiration after exposure to chronic hypoxia (10%O_2 for 3 days) of ovarectomized WT females showed that the substitution of progesterone also decreased the tidal volume in moderate (10%O_2) and the first two minutes of severe (6%O_2) hypoxia (Fig. 16c) while there were again no significant changes concerning the respiratory frequency (Fig. 16b). This reduced tidal volume caused a decreased ventilation of ovarectomized WT females after progesterone substitution

compared to control-injected animals (Fig. 16a). When Progesterone was substituted in ovarectomized Tg6 females, they showed a significantly decreased ventilation already in normoxia. The same occurred in the situations of moderate and severe hypoxia. This reduction of ventilation was due to a decreased tidal volume rather than respiratory frequency (Fig. 16d – f). These data suggest that progesterone is also participating in the blunted VAH of Tg6 females.

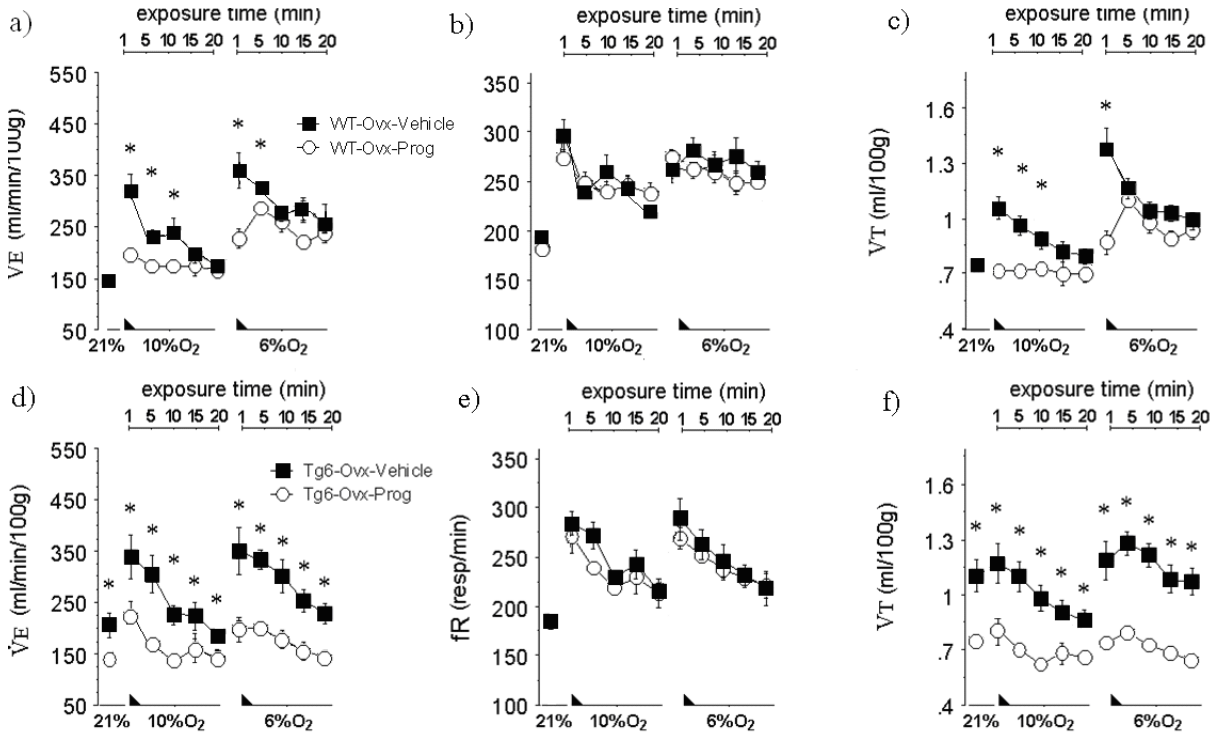

Figure 16. Minute ventilation and HVR after chronic hypoxia in ovarectomized WT and Tg6 females after substitution of progesterone.
The graphs show ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) in ovarectomized WT with NaCl injections (vehicles) and ovarectomized WT with progesterone substitution (0.1mg i.p./day for 7 days) under conditions of normoxia, moderate ($10\%O_2$) and severe ($6\%O_2$) hypoxia. The same is shown for ovarectomized Tg6 females (d) – f)). *$p<0.05$ WT-Ovx-Vehicle vs. WT-Ovx-Prog and Tg6-Ovx-Vehicle vs. Tg6-Ovx-Prog; Animals per group = 7-9.

We also measured the transient ventilatory decline in response to a brief period of hyperoxia ($100\%O_2$, Dejours test) in ovarectomized WT and Tg6 females after preexposure to chronic hypoxia ($10\%O_2$ for 3 days) in presence of progesterone (0.1mg i.p./day for 7 days). These measurements show that there were no significant differences neither in ventilation (Fig. 17a), respiratory frequency (Fig. 17b) nor tidal

volume (Fig. 17c) in response to hyperoxia in WT compared to Tg6 females. These data suggest that progesterone does not affect the sensitivity of carotid bodies to changes in the PaO_2 of arterial blood.

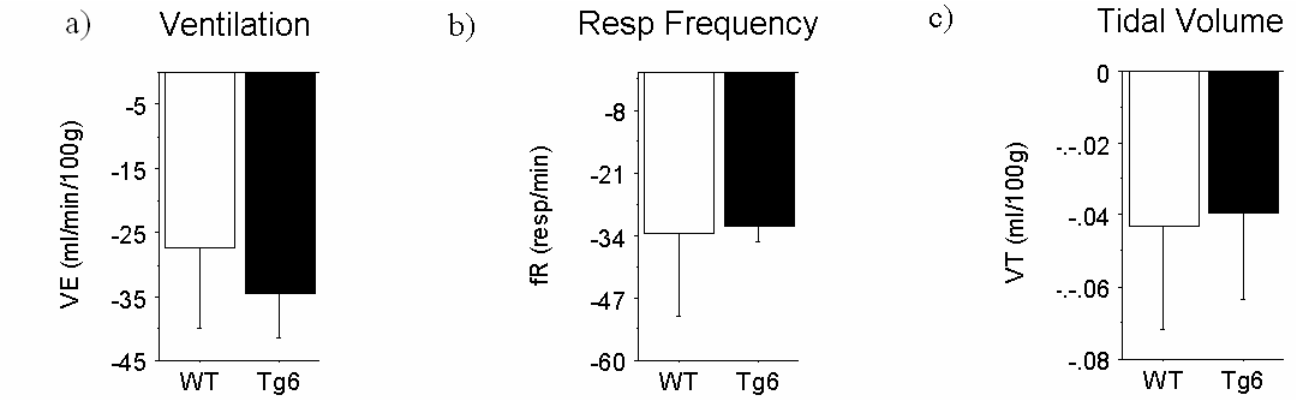

Figure 17. Dejours test after chronic hypoxia in ovarectomized WT and Tg6 females with substituted progesterone.
Decline of ventilation (VE; a)), respiratory frequency (fR; b)) and tidal volume (VT; c)) upon transition from 21% to 100% O_2 (Dejours test). The magnitude of the transient ventilatory decline was calculated as the difference between baseline and hyperoxic respiratory parameters. The experiment was performed in urethane anaesthetized ovarectomized WT and Tg6 females after substitution of progesterone (0.1mg i.p./day for 7 days). *p<0.05; animals per group = 7-8.

6. DISCUSSION

In the present work we demonstrate that plasma Epo, by interacting with female sexual steroids, abolishes the ventilatory acclimatization to chronic hypoxia (VAH) of mice.

In recent work the measurement of ventilation of male Tg21 mice, that overexpress Epo in brain only, showed in comparison to WT mice (i) a higher hypoxic ventilatory response (HVR) to severe acute hypoxia (6%O_2), (ii) and an enhanced HVR to moderate (10%O_2) and severe (6%O_2) hypoxia after acclimatization to reduced oxygenation (VAH; 3days at 10%O_2) (Soliz, Joseph et al. 2005). Moreover, following bilateral transection of the carotid sinus nerve (chemodenervation), that uncouples carotid bodies from the brain, Tg21 mice adapted their ventilation to acute severe hypoxia, while chemodenervated WT developed a life-threatening apnea (Soliz, Joseph et al. 2005). In parallel, it was demonstrated in WT mice that intracerebroventricular infusion of soluble EpoR, an antagonist to the binding of Epo to the Epo receptor (EpoR), abolished the VAH (Soliz, Gassmann et al. 2007). In line with these results it was shown that respiratory centers in the brainstem express the corresponding receptor for Epo (Soliz et al., 2005). All these data show that cerebral synthesized Epo is a physiologically relevant factor during hypoxic ventilation and in the process of acclimatization to chronic hypoxia. Ventilatory measurements of male WT animals intravenously injected with rhEpo revealed that Epo altered the ventilatory pattern. Epo-injected animals showed higher respiratory frequency but lower tidal volume than saline injected controls upon severe (6%O_2) hypoxia (Soliz, Joseph et al. 2005). Similar experiments were performed in Tg6 mice that show Epo overexpression in brain and lung tissue, aiming to examine the impact of high Epo levels and excessive erythrocytosis on ventilation in acute and chronic hypoxia. The results showed that while the minute ventilation was similar to WT control animals, the ventilatory pattern changed dramatically (respiratory frequency in Tg6 mice was significantly higher while tidal volume decreased) (Soliz, Soulage et al. 2007). These data imply that carotid bodies are activated by plasma Epo. In summary, the results obtained from these three different animal models (Tg21, Tg6 and Epo-injected WT)

provided convincing evidence that Epo regulates hypoxic ventilation by interacting at central (brainstem) and peripheral (carotid bodies) levels (Soliz, Joseph et al. 2005). Because hypoxic ventilation has been reported to be gender-dependent (Joseph, Soliz et al. 2000; Joseph, Soliz et al. 2002) minute ventilation and HVR of female mice have recently been measured (Soliz, Thomsen et al. 2009). In agreement with observations in human beings data showed that female mice cope better with hypoxic conditions compared to males. When the same experiments were repeated with Tg21 and Tg6 female mice, data demonstrated that systemic and cerebral Epo impacts the acute HVR in a gender-dependent manner(Soliz, Thomsen et al. 2009).

The present study was designed to investigate the impact of high Epo levels in the ventilatory acclimatization to chronic hypoxia of female mice. Experiments were performed in Tg6 female mice that constitutively overexpress Epo in brain (26 fold over WT) and in lung tissue (12 fold over WT) leading to hematocrit values up to 90% (Ruschitzka, Wenger et al. 2000; Wagner, Katschinski et al. 2001; Heinicke, Baum et al. 2006). Surprisingly, in contrast to corresponding controls (WT female and Tg6 male mice) Tg6 female mice were not able to reach VAH after exposure to chronic hypoxia (3 days at 10% O_2). To investigate whether the respiratory areas in the brainstem or the carotid bodies were involved in the observed abolished VAH, we performed ventilatory measurements in two different animal models: (i) Tg6 females after bilateral transection of the carotid sinus nerves (chemodenervation), leading to inhibited transmission of information from carotid bodies to the brainstem, and (ii) Tg21 females, that overexpress Epo in brain only. Our results showed similar VAH between chemodenervated Tg6 and WT females, and also between Tg21 females and corresponding WT female controls. Thus, we concluded that the cerebral overexpression of Epo was not responsible for the abolished VAH observed in untreated Tg6 females.

Apart from central regulation, we also performed experiments to investigate the implication of plasma Epo in the observed blunted VAH of female Tg6 mice. Carotid bodies are organs specialized in the detection of reduced oxygen content in the arterial blood, and corresponding transmission of this information to the respiratory centers of the brainstem (Kumar 2007; Ward 2008). During embryogenesis, carotid bodies develop from the ectodermal layer (Kondo, Iwanaga et al. 1982), implying that carotid glomus cells, similarly as cerebral cells, express receptor for Epo (EpoR). Indeed, previous reports using immunohistochemistry showed a dense staining of

EpoR in carotid body cells (Soliz, Joseph et al. 2005). We verified these data by showing that Epo bound to carotid glomus cells.

Carotid bodies have been reported to be one of the major sites for gender-dependent control of ventilation (Pequignot, Spielvogel et al. 1997; Tatsumi, Pickett et al. 1997; Joseph, Soliz et al. 2002). Moreover, it is known that ovarian steroids are potent stimulants of the peripheral respiratory centers (Bayliss, Millhorn et al. 1987; Bayliss, Cidlowski et al. 1990; Hannhart, Pickett et al. 1990; Tatsumi, Pickett et al. 1997). Accordingly, female sexual steroidreceptors are present in carotid bodies (Joseph, Doan et al. 2006). As the blunted VAH occurs exclusively in Tg6 female mice, we hypothesized that the interaction of plasma Epo with female sexual steroids (estradiol and/or progesterone) at the carotid bodies is the cause of this phenomenon. Coherently, we observed that after ovariectomy Tg6 females were able to recover the ventilatory acclimatization. Considering the fact that, as mentioned before, ovarian steroids are potent stimulants of the respiratory system (Bayliss, Millhorn et al. 1987; Bayliss, Cidlowski et al. 1990; Hannhart, Pickett et al. 1990; Tatsumi, Pickett et al. 1997) it was not surprising that ovarectomized WT females in contrast to ovarectomized Tg6 females decreased ventilation after preexposure to chronic hypoxia (10%O_2 for 3 days).

In a next step we wanted to investigate whether estradiol or progesterone participate in the abolishment of VAH of Tg6 females. To this end, we followed a hormonal replacement protocol in ovarectomized Tg6 and WT females. As expected, the ventilatory measurements showed that the substitution of estradiol in ovarectomized Tg6 females blunted the VAH due to a decrease in tidal volume, rather than respiratory frequency. When similar experiments were performed in ovarectomized WT females, we observed that WT females did not show ventilatory differences after substitution of estradiol compared to control injections. In line with these results, the carotid body sensitivity to oxygen changes in arterial blood (Dejous test) showed a lower sensitivity of the transgenic peripheral chemoreceptors when estradiol was substituted in ovarectomized Tg6 females. These results constitute a clear evidence that Epo and estradiol undergo a deleterious interaction in the carotid body exposed to chronic hypoxia.

Interestingly, however, similar results were found when, instead of estradiol, progesterone was substituted in ovarectomized Tg6 females. This observation was

rather surprising, as several papers reported that progesterone is increasing ventilation (Takano 1984; Masuda, Ohyabu et al. 2001; Lefter, Morency et al. 2007).
Several studies showed that ovarian steroids can influence the expression of hypoxia-inducible genes, such as Epo, vascular endothelial growth factor, endothelial nitric oxide synthase and hypoxia-inducible factor-1 (Bausero, Ben-Mahdi et al. 2000; Mukundan, Resta et al. 2002; Mukundan, Kanagy et al. 2004; Mukundan, Resta et al. 2004; Shimizu and Miyamoto 2007). Concerning ventilation, estradiol and progesterone are known as potent stimulants of the central (Bayliss, Millhorn et al. 1987; Bayliss, Cidlowski et al. 1990; Bayliss and Millhorn 1992; Lefter, Morency et al. 2007) and peripheral (Hannhart, Pickett et al. 1990; Tatsumi, Pickett et al. 1997; Joseph, Soliz et al. 2002; Lefter, Morency et al. 2007) ventilatory system. Several studies reported that minute ventilation and the hypoxic ventilatory responsiveness in pregnant women are increased compared to nonpregnant women (Moore, McCullough et al. 1987). Increased ventilation and HVR can also be observed in the luteal phase of the menstrual cycle, where progesterone is the predominant hormone (Goodland, Reynolds et al. 1953; Schoene, Robertson et al. 1981; Takano 1984). In addition, when progestin was injected in male volunteers, augmentation of ventilation and HVR was also reported (Skatrud, Dempsey et al. 1978; Zwillich, Natalino et al. 1978). It was proposed that estradiol may potentiate the stimulatory effect of progestin on ventilation (Brodeur, Mockus et al. 1986) most probably by inducing progesterone receptors (Sherman, Corvol et al. 1970). In addition, different studies in humans showed that female sexual steroids are involved in numerous hypoxia-associated sicknesses and syndromes with men having higher susceptibility to these pathologies (Leon-Velarde, Ramos et al. 1997; Joseph, Soliz et al. 2000). As such, chronic mountain sickness (CMS) predominantly occurs in men and postmenopausal women (Leon-Velarde, Ramos et al. 1997; Joseph, Soliz et al. 2000).
Our observation that progesterone decreases ventilation in ovarectomized WT females stands in contrast to all these studies. However, when we injected the same amount of progesterone to WT males we also observed a significant decrease of ventilation compared to control-injected males (unpublished data). Unfortunately, from the performed experiments it is not possible to explain this result, however we suggest that this is due to the high concentration of progesterone that was administrated to the animals. In the next future we plan to perform dose-dependent hormonal stimulation in PC12 cells incubated with Epo to elucidate this issue.

In conclusion, our data indicate that in combination with estradiol and progesterone Epo has a deleterious impact on the ventilatory acclimatization of mice. We showed that Tg6 females have a blunted ventilatory acclimatization to chronic hypoxia, and that this blunted VAH can be abolished by ovariectomy. Moreover we demonstrated that this interaction occurs on the peripheral respiratory system and that the respiratory areas in the brainstem are not implicated in this phenomenon. Keeping in mind that an adequate chemoreflex function is a key component of the ventilatory acclimatization to hypoxic environments, our findings suggest that the Epo interaction with sexual female hormones has important clinical implications, especially in patients suffering from chronic obstructive pulmonary diseases, and in several million people worldwide that are permanently exposed to high altitude.

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